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Dichotomy of two $CD8^+$ lymphocyte subsets in HIV infection. Depletion of $CD8^+CD3^-$ and expansion of $CD8^+CD3^+$ subsets: consequence on the CD4/CD8 ratio

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SUMMARY

In order to highlight the underlying mechanism(s) of the CD8 lymphocyte expansion in the HIV infection, two distinct CD8 subsets were analysed: T $CD8^{bright+}$ $CD3^+$ with MHC-restricted activity, and non-T $CD8^{dim+}$ $CD3^-$, which performs natural killer (NK) activity. It consists of a cross-sectional study including 168 HIV-infected patients (74 CDC stage II, 48 CDC stage III and 46 CDC stage IV) compared among them and to 60 healthy individuals. We observed an expansion of $CD8^+CD3^+$ cells which masks a depletion of $CD8^+CD3^-$. The comparative study showed that the expansion of the $CD8^+CD3^+$ is relatively higher than that of total $CD8^+$ lymphocytes and that the depletion of the $CD8^+CD3^-$ subset is severe, begins early and remains constant through the HIV progression. The comparison of CD4/CD8 and CD4/$CD8^+CD3^+$ ratios showed that the latter could possibly be a better indicator in the HIV infection. The mechanism of inverted CD4/CD8 ratio in healthy individuals was also clarified. The $CD8^+CD3^+$, $CD8^+CD3^-$ and CD4/$CD8^+CD3^+$ parameters would be more specific markers than total CD8 and CD4/CD8 ratio especially in therapy trials.

Keywords HIV infection lymphocyte impairing CD8 subpopulations CD4/CD8 ratio flow cytometry

INTRODUCTION

Infection by HIV results in the severe cellular and humoral immune dysfunctions known as AIDS. A depletion of $CD4^+$ lymphocytes and an expansion of $CD8^+$ cells are prominent features of this disease (Melbye *et al.*, 1986; Cooper *et al.*, 1988). The infection of $CD4^+$ cells leads to their death (Klatzmann *et al.*, 1984) and would be responsible for some of the defects in the immunological response. However, the interpretations of the increase of the $CD8^+$ lymphocyte population are controversial; it was hypothetically attributed to the immunosuppression (Lewis *et al.*, 1985) or described as an appropriate host response to infection of malignancy in patients with AIDS or ARC (Stites *et al.*, 1986). Besides, the expansion of $CD8^+$ lymphocytes was seldom correlated with other features of the infection such as the antigenaemia (Andrieu *et al.*, 1989), the seroconversion and the presence of HIV-specific antibodies (Cooper *et al.*, 1988); in addition, such correlations were weak, often controversial, and concerned particular stages of the infection.

The $CD8^+$ lymphocytes are heterogeneous in subphenotypes and functions; therefore, further dissection in subpopulations is essential. It would delineate the mechanism(s) of the total CD8 lymphocyte expansion in AIDS through the identification of the increased CD8 subset and would highlight multiple unexplained and/or contradictory observations reported in the literature on this increase. As there are no unique monoclonal antibodies (MoAbs) that could correlate CD8 subsets to exact functions, we analysed two subsets, $CD8^+CD3^+$ and $CD8^+CD3^-$, in the HIV infection. These phenotypes supply a reliable means to clearly distinguish two different CD8 subsets using three main characteristics: the cell type; the activity *in vitro*; and the CD8 surface antigen density. Whereas the $CD8^+CD3^+$ subset includes T cells which exhibit MHC-restricted activity and express high-density CD8 ($CD8^{bright+}$), the $CD8^+CD3^-$ subset includes non-T lymphocytes which perform natural killer (NK) activity and express the CD16 NK marker and low density CD8 ($CD8^{dim+}$) (Lanier *et al.*, 1983; Perussia, Fanning & Trinchieri, 1983; Mansour *et al.*, 1990c).

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SUBJECTS AND METHODS

Five millilitres of venous blood were collected on EDTA from 168 HIV-infected patients at the following stages of infection, according to the classification of the Centers for Disease

Control (CDC); 74 CDC II, 48 CDC III and 46 CDC IV (eight IV.A, three IV.B, 18 IV.C1, five IV.C2, three IV.E and nine IV.D) (Centers for Disease Control, 1986). The patients' mean aged was 34 years (range 21–61). Blood was also taken from 60 healthy controls (mean age 38 years, range 22–59).

Lymphocyte preparation

Lymphocytes were prepared for cytometry analysis using a whole blood lysis technique (Mansour *et al.*, 1990b). In general, 2 ml were lysed using 40 ml of a formaldehyde-free lysing buffer (Ortho) and washed in a pre-cooled (4°C) phosphate-buffered saline (PBS) (BioMérieux) supplemented with 0·1% sodium azide (PBS-a; pH 7·4). In cases of lymphopenia, we used a higher volume of blood to enrich the lymphocyte suspension for appropriate flow cytometry analysis. In all cases, cells were resuspended in 2 ml of PBS-a and stored at 4°C until labelling.

Immunofluorescence labelling

MoAbs were used in simple and dual immunofluorescence; these included the OKT8–^{FITC} (CD8), OKT4–^{FITC} (CD4), OKT3–^{PE} (CD3) (Orthomune) and Leu-M3–^{FITC} (CD14) (Becton Dickinson) MoAbs. The following phenotypes were analysed: $CD14^+$, $CD4^+$, $CD8^+$, $CD8^+CD3^+$, $CD8^+CD3^-$. One-hundred microlitres of the cell suspension were incubated with the MoAbs at 4°C for 30 min. Labelled cells were washed in 5 ml of PBS-a, diluted to a final volume of 1 ml in PBS-a and stored at 4°C until analysis.

Cytometric analysis

The total leucocyte count (cells/mm^3) was obtained using the Baker 8000 haemocytometer. The proportion of lymphocytes was determined using the Ortho Spectrum III cytometer interfaced to a 2140 analyser (Ortho) and a fluorescence compensated dual logarithmic amplifier module (DLM). Lymphocytes were distinguished from monocytes and granulocytes on the basis of their forward *versus* right angle light scatter and the exclusion of $CD14^+$ monocytes. The DLM allowed the correction of spectral overlap between the FITC and the PE and provided logarithmic displays of fluorescence (3·5 decades). It is worth noting that the logarithmic fluorescence analysis is essential for the analysis of $CD8^{dim+}$ lymphocytes. The count of each lymphocyte subset was then determined: total leucocyte count × lymphocyte proportion × subset proportion. The final count corresponds to the mean of duplicate analysis.

Statistical analysis

Results are given as mean values ± s.e.m. Inter-group statistical significance was determined using the unpaired Student's *t*-test and intra-group using the Mann–Whitney *U*-test. Probability values lower than 0·05 were considered significant. Absolute numbers and percentages were analysed simultaneously to distinguish between a selective subset depletion from a general alteration such as lymphopenia.

RESULTS

Preliminary results

The total lymphocyte count showed no alterations in CDC stages II and III in comparison to the control group, whereas the CDC stage IV showed a significant lymphopenia (Table 1). The CD4 lymphocyte population was diminished in all patients groups, and the lowest mean count was in the CDC IV group (Table 1).

Table 1. Comparative enumeration of total and $CD4^+$ lymphocytes (only CDC stage IV shows a significant lymphopenia, but all CDC stages show a depletion of $CD4^+$ lymphocytes)

	Controls ($n=60$)	CDC II ($n=74$)	CDC III ($n=48$)	CDC IV ($n=46$)
Total lymphocytes	1743 ± 57	1780 ± 64 (NS)	1728 ± 82 (NS)	1398 ± 113 ($P \leqslant 0{\cdot}01$)
CD4 lymphocytes	785 ± 27	476 ± 27 ($P \leqslant 0{\cdot}001$)	421 ± 38 ($P \leqslant 0{\cdot}001$)	276 ± 40 ($P \leqslant 0{\cdot}001$)

NS, not significant.

$CD8^+$ population and $CD8^+CD3^+$ versus *$CD8^+CD3^-$ subpopulation counts*

In comparison to the control group, absolute counts and percentages of total $CD8^+$ lymphocytes in all HIV-infected groups showed statistically significant increase. However, the subset division showed two patterns of evolution within CD8 cells; the $CD8^+CD3^+$ subset was increased significantly, whereas the $CD8^+CD3^-$ was severely depleted (Table 2).

For the comparison of patient groups among them (Table 2), it is pertinent to divide results into two categories. In the first category, we compared CDC stage II with CDC stage III and observed no statistically significant difference for all phenotypes. In the second category, we compared both CDC stages II and III with the CDC stage IV; the latter stage showed further significant increase only in percentages of $CD8^+$ and $CD8^+CD3^+$ cells, which indicates a continuous expansion of these cells through the evolution to AIDS, and no significant decrease of the $CD8^+CD3^-$ subset, which indicates that the severe depletion is definitive throughout the infection evolution. It is worth noting that the alterations of absolute counts do not reveal the same outcome as percentages due to the general lymphopenia in AIDS (Table 1).

CD4/CD8 versus *$CD4/CD8^+CD3^+$ ratios*

The comparison of the CD4/CD8 with $CD4/CD8^+CD3^+$ ratio within each group showed statistically significant difference only in the control group (Table 3). This difference is due to the effect of the $CD8^+CD3^-$ subset in the control group but not in the HIV-infected groups in which this subset is depleted. This would indicate higher specificity of the $CD4/CD8^+CD3^+$ ratio notably in the early stage of the infection. In fact, the statistical comparison of patient groups among them showed no significant difference for both ratios (data not shown) which indicates similar informativity of either ratio in the disease evolution. However, the decrease of the $CD4/CD8^+CD3^+$ ratio in patient groups with respect to controls is more spectacular than that of the CD4/CD8 ratio.

DISCUSSION

In general, investigators designate the $CD8^+$ population as T suppressor/cytotoxic lymphocytes. Nevertheless, this classifi-

Table 2. Analysis of the total $CD8^+$ lymphocyte population and $CD8^+CD3^+$ and $CD8^+CD3^-$ subsets

	Controls ($n=60$)	CDC II ($n=74$)	CDC III ($n=48$)	CDC IV ($n=46$)	C *versus* II	C *versus* III	C *versus* IV	II *versus* III	II *versus* IV	III *versus* IV
$CD8^+$	541±27	991±49	944±47	859±67	$P\leqslant 0.001$	$P\leqslant 0.001$	$P\leqslant 0.001$	NS	NS	NS
(cells/mm^3, %)	31±0·8	55±1·3	56±1·7	63±1·7	$P\leqslant 0.001$	$P\leqslant 0.001$	$P\leqslant 0.001$	NS	$P\leqslant 0.001$	$P\leqslant 0.001$
$CD8^+CD3^+$	393±21	903±47	877±46	810±65	$P\leqslant 0.001$	$P\leqslant 0.001$	$P\leqslant 0.001$	NS	NS	NS
(cells/mm^3, %)	22±0·8	51±1·4	52±1·8	59±1·6	$P\leqslant 0.001$	$P\leqslant 0.001$	$P\leqslant 0.001$	NS	$P\leqslant 0.001$	$P\leqslant 0.001$
$CD8^+CD3^-$	125±10	53±5	48±6	29±3	$P\leqslant 0.001$	$P<0.001$	$P\leqslant 0.001$	NS	$P\leqslant 0.001$	$P\leqslant 0.001$
(cells/mm^3, %)	7·1±0·4	3·1±0·3	2·6±0·3	2·5±0·3	$P\leqslant 0.001$	$P\leqslant 0.001$	$P\leqslant 0.001$	NS	NS	NS

Results are mean±s.e.m. The comparison of data between patient groups and controls showed highly significant increase of total $CD8^+$ and a dichotomy of subsets in all CDC stages. The $CD8^+CD3^+$ subset is highly increased and the $CD8^+CD3^-$ subset is severely depleted. In the comparison of patient groups among them, the simultaneous analysis of absolute counts and percentages shows off the difference between the specific subset alteration in CDC IV from a general alteration due to the lymphopenia.

NS, not significant.

C, controls.

Table 3. Comparison of CD4/CD8 to $CD4/CD8^+CD3^+$ ratio: there is a significant difference only in the control group due to the effect of the non-T $CD8^+CD3^-$ subset

	Controls ($n=60$)	CDC II ($n=74$)	CDC III ($n=48$)	CDC IV ($n=46$)
CD4/CD8 ratio	1·45	0·48	0·44	0·32
$CD4/CD8^+CD3^+$ ratio	2	0·53	0·48	0·34
Mann–Whitney *U*-test	$P\leqslant 0.001$	NS	NS	NS

NS, not significant.

cation is somewhat rough, since 26–30% of the $CD8^+$ lymphocytes are non-T cells, express the CD16 NK phenotype and exhibit non-MHC-restricted activity (Lanier *et al.*, 1983; Perussia *et al.*, 1983; Mansour *et al.*, 1990c). This proportion is significant and should be taken into account in the analysis of the CD8 compartment of lymphocytes. In addition, T and non-T CD8 subsets are likely to behave differently during infections, since they represent different functional characteristics and most probably undergo different regulations. In this scope, we analysed the total CD8 cell population, T CD8 ($CD8^+CD3^+$) and non-T CD8 ($CD8^+CD3^-$) lymphocytes in HIV infection.

As expected, the total CD8 cell count is increased in all stages, a feature of HIV infection that is already well documented (Kornfeld *et al.*, 1982; Fahey *et al.*, 1984; Lewis *et al.*, 1985; Stites *et al.*, 1986). However, the subdivision of CD8 cells showed that the expansion affects only the $CD8^+CD3^+$ subset. With respect to controls, the rise of this subset is relatively higher than that of total CD8 cells through the dissociation from the decreased $CD8^+CD3^-$ subset (Table 2). The $CD8^+CD3^+$ subset is likely to be heterogeneous functionally as well as phenotypically and therefore to represent other changes. Reliable markers that correlate $CD8^+CD3^+$ subpopulations with specific functions would be useful for further refining of analysis. Besides, the depletion of $CD8^+CD3^-$ lymphocytes begins in the asymptomatic stage of the infection, and is too severe at this stage for any aggravation through the infection evolution to be spectacular. So the subdivision into $CD8^+CD3^-$ and $CD8^+CD3^+$ subsets reduced the heterogeneity of CD8 lymphocytes and showed off opposite patterns of evolution through the disease. This would provide complementary informations more specific to HIV disease than that provided by total CD8 population. For instance, the depletion of $CD8^+CD3^-$ lymphocytes may explain the decrease of total CD8 observed in some HIV-infected subjects (Melbey *et al.*, 1986; Cooper *et al.*, 1988) at the initial stage of the infection (Cooper *et al.*, 1988). Also, the correlation of either subset with other features of the disease would possibly provide supplementary information useful to study the specificity of the rise in CD8 cells in HIV disease and its progression. This subdivision would also be interesting in other infections such as cytomegalovirus (Carney *et al.*, 1981; Drew *et al.*, 1985) and Epstein–Barr virus (de Waele, Thielemans & van Camp, 1981) in which the $CD8^+$ cell count is impaired.

Earlier data from our laboratory showed that $CD8^+CD3^-$ cells express the CD16 NK cell marker (Mansour *et al.*, 1990c) and that the depletion of $CD8^+CD16^+$ lymphocytes is responsible for a decrease in the NK cell compartment (Mansour, Doinel & Rouger, 1990a). In this report, however, we stress the subdivision of CD8 cells into $CD8^+CD3^-$ and $CD8^+CD3^+$ subsets and the analysis of $CD4/CD8^+CD3^+$ ratio. In fact, $CD3^+$ and $CD8^+$ lymphocytes are assessed routinely in the HIV infection using the simple immunofluorescence assay; instead, the two-colour immunofluorescence, which requires the same quantities of MoAbs and technical steps, allows one to estimate $CD3^+$, $CD8^+$, $CD8^+CD3^-$ and $CD8^+CD3^+$ phenotypes and $CD4/CD8^+CD3^+$ ratio and to gain valuable information.

In view of these results, it would be interesting to reconsider the CD4/CD8 ratio concept through the analysis of the $CD4/CD8^+CD3^+$ ratio. The latter might improve the specificity by decreasing the heterogeneity of the populations under study, notably by excluding the effect of the $CD8^+CD3^-$ subset decrease. In fact, as reported by Melbye *et al.* (1986), the decline of the CD4/CD8 ratio is probably more sensitive than the alterations of either CD4 and CD8 lymphocytes separately, since it is affected by the changes of both cell populations. In contrast, Henderson *et al.* (1988) observed that the CD4/CD8 ratio is less meaningful than the absolute numbers of cells. This discordance may be due to the fact that some laboratories

normally exclude the $CD8^+CD3^-$ ($CD8^{dim+}$) in their gating during cytometry analysis. In fact, the CD4/CD8 ratio consists of $CD4^+$, mainly T cells (data not shown), over T ($CD8^+CD3^+$) and non-T ($CD8^+CD3^-$) cells. Consequently, a decrease of the latter subset would impair the CD4/CD8 ratio independently of the T cell context and decreases its specificity as it has opposite pattern of evolution to the $CD8^+CD3^+$ subset. As shown in Table 3, the decrease of the CD4/$CD8^+CD3^+$ ratio is more spectacular than that of CD4/CD8. The follow up of the former, in the disease's natural history and therapeutic trials, would most probably be more interesting than the latter.

The CD8 subdivision is also useful to highlight the inversion of CD4/CD8 ratio in healthy individuals. In this study, for instance, seven out of 60 healthy controls had inverted CD4/CD8 ratio (data not shown). These seven controls have CD4 cell count within normal ranges but an increased CD8 count. However, the subset subdivision showed constant $CD8^+CD3^+$ counts, but high $CD8^+CD3^-$ counts paralleled a significant NK compartment.

This cross-sectional study supplies a body of argument that the analysis of the $CD8^+CD3^+$ and $CD8^+CD3^-$ subsets and the CD4/$CD8^+CD3^+$ ratio would supply valuable information in healthy individuals, in the natural history of HIV disease and in therapeutic trials.

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